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Biocatalytic synthesis of uridine 5′-diphosphate *N*-acetylglucosamine by multiple enzymes co-immobilized on agarose beads

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Recombinant *N*-acetylglucosamine kinase, pyruvate kinase, *N*-acetylglucosamine phosphate mutase, uridine 5′-diphosphate *N*-acetylglucosamine pyrophosphorylase, and inorganic pyrophosphatase were overexpressed in *E. coli* and co-immobilized on agarose beads for the practical synthesis of uridine 5′-diphosphate *N*-acetylglucosamine.

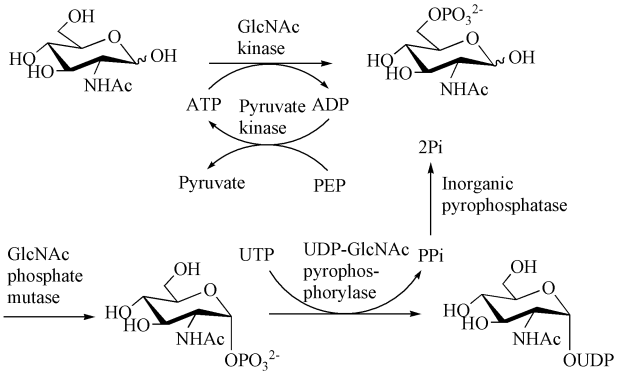
Efficient synthesis of oligosaccharides has recently drawn much attention due to their distinct physiological functions and pharmaceutical values. Leloir glycosyltransferases catalyze the straightforward synthesis of oligosaccharides with stereo- and regioselectivity, but the practical application relies highly on the availability of expensive sugar nucleotides. Although the sugar nucleotides can be prepared chemically,[1] the most economic route is enzymatic synthesis by following the biosynthetic pathway.

Uridine 5′-diphosphate *N*-acetylglucosamine (UDP-GlcNAc) is an essential metabolite in the biosyntheses of peptidylglycan, chitin, glycosaminoglycans and glycoproteins. The biosynthesis of UDP-GlcNAc has been extensively studied, which is the normal 6-phosphate to 1-phosphate, then to sugar nucleotide pathway. Enzymatic[2] and fermentative[3] approaches have been developed for the production of UDP-GlcNAc. They all make use of the natural biosynthetic pathway with some degree of modification, and have obtained desirable yields of sugar nucleotide. However, most of these biocatalysts are not cheap or stable enough as versatile reagents in chemoenzymatic synthesis. In addition, it is extremely hard to recover them from the reaction mixture for repeated usage. Immobilization of enzymes, on the other hand, possesses many advantages such as stabilization, reusability of the enzymes and simplified isolation of the product, thereby offering the likely solution to those problems. Actually, some of the immobilized enzymes have been used in the production of glycoconjugates.[4]

We describe here the expression of five enzymes involved in UDP-GlcNAc biosynthetic pathway, their co-immobilization and stabilization on agarose beads, which are capable of synthesizing UDP-GlcNAc. In this system (Scheme 1), *N*-acetylglucosamine (GlcNAc) is first phosphorylated by GlcNAc kinase (GlcK, *C. albicans*).[5] Then GlcNAc phosphate mutase (Agm1, *S. cerevisiae*)[6] converts GlcNAc 6-phosphate (GlcNAc 6-P) to GlcNAc 1-phosphate (GlcNAc 1-P), which is subsequently uridylated to form UDP-GlcNAc by a truncated UDP-GlcNAc pyrophosphorylase (GlmU, *E. coli*).[7] The resulting ADP can be reconverted to ATP by pyruvate kinase (PykF, *E. coli*)[8] with the consumption of one equivalent of phosphoenolpyruvate (PEP). The by-product pyrophosphate (PP_i) is finally hydrolyzed by inorganic pyrophosphatase (PPA, *E. coli*)[9] as a driving force.

The genes encoding each enzyme were amplified by polymerase chain reaction (PCR), cloned into pET15b vector, and expressed at 30 °C in *E. coli* BL21(DE3) with hexahistidine (6 × His) tags. SDS-PAGE indicated that all the proteins were overexpressed in soluble form (data not shown). Since all the enzymes have a 6 × His tag, they can be easily immobilized by immobilized metal affinity chromatography (IMAC), which is widely used to facilitate the purification of recombinant proteins. After the disruption of cells by brief sonication, cell lysate mixture was prepared with roughly equal activity of individual enzyme. The UDP-GlcNAc production beads were then obtained by incubating the cleared cell lysate mixture with Ni–NTA agarose resins at 4 °C for 30 min, and washing with Tris-HCl buffer (50 mM, pH 7.5) to remove unbound proteins. Enzymatic activity assays indicated that more than 80% of the recombinant enzymes were immobilized on the agarose beads (Table 1).

Gram-scale synthesis of UDP-GlcNAc was conducted at 30 °C in a mixture (100 mL) containing GlcNAc (440 mg, 2 mmol), PEP (380 mg, 2 mmol), ATP (55 mg, 0.1 mmol), UTP (1.1 g, 2 mmol), Glc-1,6-dP_i (1.4 mg, 40 μmol), $MgCl_2$ (10 mM) and KCl (50 mM) in Tris-HCl (50 mM, pH 7.0). The reaction was initiated by the addition of 10 mL multiple enzyme beads. The progress of the reaction was monitored by capillary electrophoresis (Fig. 1). A time course study revealed that the reaction reached saturation within 20 h (Fig. 2). After the reaction had completed, the beads were recovered from the mixture by centrifugation (2000*g*, 5 min) and washed with Tris-HCl (50 mM, pH 7.5) for another reaction. The supernatant was applied to a DEAE-cellulose ion-exchange column to separate the product. UDP-GlcNAc was further purified by a Sephadex



Scheme 1 Multiple-enzyme system for the synthesis of UDP-GlcNAc.

Table 1 Expression of the enzymes in *E. coli* BL21(DE3) and their immobilization on Ni–NTA agarose beads

Enzyme	Crude extract enzyme activity (mU mL^{-1})[ab]	Immobilized enzyme activity (mU mL^{-1})[ab]	Yield[c] (%)
GlcK	22.4	492	88
Agm1	18.1	412	91
GlmU	28.6	615	86
PykF	30.0	633	83
PPA	24.3	516	83

[a] One unit (U) of enzyme activity is defined as the amount of enzyme that produces 1 μmol of product per min at 30 °C. [b] The recombinant enzymes from 500 mL cell lysate mixture were immobilized on 20 mL Ni–NTA agarose resins. [c] Immobilization yield is expressed as the ratio of immobilized enzyme activity to that from cell crude extract.

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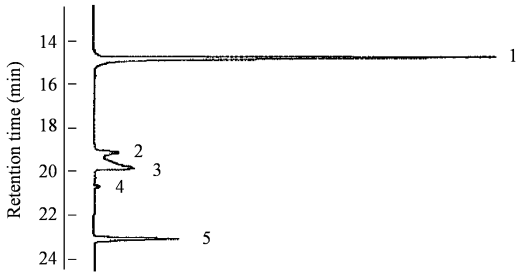

Fig. 1 Capillary electrophoresis profiles of the enzymatic reaction at 16 h. Electrophoresis was run in 75 μm × 50 cm (40 cm to detector) bare fused silica capillary, under 22 kV with UV detection at 262 nm. UDP-GlcNAc has a retention time of 14.7 ± 0.2 min. The identities of each peak are as follows: 1. UDP-GlcNAc; 2. ATP; 3. UTP; 4. ADP; 5. UDP.

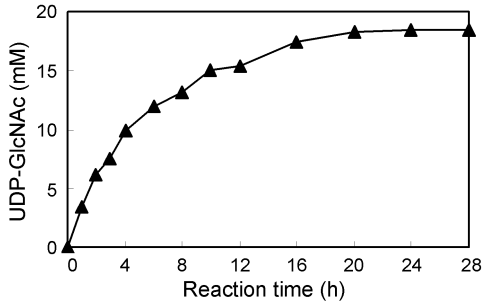

Fig. 2 The time course for UDP-GlcNAc production by multi-immobilized enzymes. Reaction mixture consisted of 20 mM GlcNAc, 20 mM UTP, 1 mM ATP, 40 μM Glc-1,6-dP_i, 20 mM PEP, 10 mM $MgCl_2$, 50 mM KCl and 50 mM Tris-HCl (pH 7.0) in a total volume of 100 mL.

G-15 gel filtration column with water as the mobile phase. The product containing fractions were pooled and lyophilized to give 1.18 g sugar nucleotide. NMR spectroscopy and mass spectrometry (ESI-MS) were utilized to identify the produced UDP-GlcNAc.†

The stability of multiple enzyme beads was demonstrated by repeated synthesis of UDP-GlcNAc. As expected, these beads were recyclable, but lost some enzymatic activities during the reactions. A 50% yield of product can still be achieved after five 20 h reaction cycles (Fig. 3). Further enzymatic assays revealed that GlcNAc phosphate mutase was the least stable enzyme on

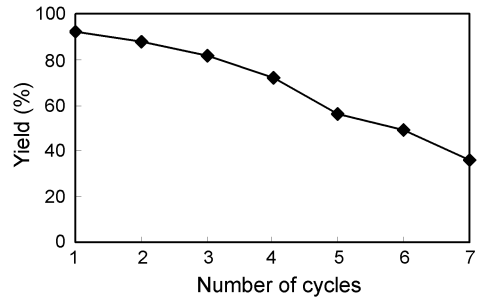

Fig. 3 Recyclability of immobilized recombinant enzymes for the synthesis of UDP-GlcNAc. All reactions were run at 30 °C for 20 h.

beads, thereby being the main reason for the observed decrease of overall yield. Addition of purified Agm1 in the reaction could partially restore the whole activity and increase the yield of UDP-GlcNAc to 78%. After repeated reactions, the deactivated enzymes were removed from the nickel beads. The agarose resins were recharged for further uses.

In summary, the UDP-GlcNAc production beads have been generated by co-immobilization of recombinant enzymes along the biosynthetic pathway of UDP-GlcNAc on Ni–NTA agarose resins. We demonstrated that these beads could be used as a common reagent in the synthesis of UDP-GlcNAc with relatively high stability. Work is in progress to introduce recombinant *N*-acetylglucosaminyltransferases into the multiple-enzyme system. These glycosyltransferases utilize UDP-GlcNAc as a substrate. The resulting UDP will be rephosphorylated to UTP by pyruvate kinase, which is then consumed to generate another molecule of UDP-GlcNAc by the immobilized enzymes. Thus, glycoconjugates can be synthesized efficiently without the purification of sugar nucleotides. It is anticipated that the so-called sugar nucleotide regeneration beads will become versatile tools for the production of glycoconjugates and their derivatives with GlcNAc residues.

Notes and references

† Uridine 5′-diphosphate *N*-acetylglucosamine (1.18 g, 92%). ^{1}H NMR (D_2O, 40 0 MHz): δ 7.90 (d, H-6″, *J* 8.1 Hz), 5.93 (d, H-1′, *J* 3.3 Hz), 5.92 (d, H-5″, *J* 8.1 Hz), 5.46 (dd, H-1, *J* 7.3, 3.3 Hz), 4.32 (m, 2H, H-2′, H-3′), 4.11–4.23 (m, 3H, H-4′, H-5′a,b), 3.72–3.97 (m, 5H, H-5, H-4, H-3, H-6a,b), 3.49 (t, 1H, H-2, *J* 8.7 Hz), 2.02 (s, 3H, Ac); ^{13}C NMR (D_2O, 100 MHz): δ 174.99, 166.50, 152.04, 141.86, 102.86, 94.74, 88.71, 83.42, 74.02, 72.23, 71.15, 69.85, 69.70, 65.22, 61.57, 60.52, 59.62, 53.94, 53.86, 22.30; ^{31}P NMR (D_2O, 162 MHz): δ −10.24, −11.91; ESI-MS (*m*/*z*): 607.76 $(M + H^+)$, 629.83 $(M + Na^+)$, 651.80 $((M - H) + 2Na^+)$, 673.80 $((M - 2H) + 3Na^+)$, 302.57 $(M - 2H)^{2-}$, 605.96 $(M - H)^-$, 627.96 $(M - 2H + Na^+)^-$.

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